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TECHNICAL MANUSCRIPT 7

APPARATUS AND METHOD FOR THE STEAM STERILIZATION OF FOOD FOR GERMFREE LABORATORY ANIMALS

An Investigation of the Practical Application of Gnotobiotic Technology to the Improvement of Breeding Colonies

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ABSTRACT

The design and preliminary use of an apparatus and method for the steam sterilization of food for germfree animals is described. The need for such a system is shown by an examination of other existing methods. In addition, the general gnotobiotic techniques and equipment used in this colony are described and the purpose of maintaining such a colony is discussed.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Dr. Melvin M. Rabstein of the Fort Detrick Animal Farm Division for his continued encouragement and guidance throughout the project; to Dr. Allen L. Stout for his aid in the initial operation of the system; to Dr. P.C. Trexler and Lobund Institute for generously providing advice, materials, and assistance throughout our work; to Mr. Roland B. Staley and his co-workers at the Fort Detrick Model Shop for the fine craftsmanship demonstrated in the fabrication of the food sterilization unit and its components; and to Mr. Charles R. Nally of the Fort Detrick welding shop for his excellent work on the jacketed adapter-plate for the autoclave.

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I. INTRODUCTION

Recent advances in gnotobiotic technology presage an increase in the use of gnotobiotic laboratory animals in medical and biological research.

In June 1960, a gnotobiotic workshop for laboratory animal breeders was held at Lobund Institute. Participants at the workshop defined three major goals in the development of a gnotobiotic program:

(a) Development of adequate apparatus and methods,

(b) Determination of useful microorganisms for association with gnotobiotic host animals, and

(c) Determination of the best associations of gnotobiotic hosts and microorganisms for various research purposes.

An important implication of the first goal is that the apparatus and methods developed must be not only adequate for the production of gnotobiotic animals but also economically feasible for use by commercial laboratory animal breeders.

Another important result of the workshop was the realization by the participants that a nucleus of gnotobiotic stock could be used to improve the quality of their existing animal colonies.

Staff members of the Animal Farm, U.S. Army Biological Laboratories, Fort Detrick, Maryland, participated in the Lobund workshop. The Fort Detrick Animal Farm has been in operation since 1944. For the past 12 years, the farm has maintained specific-pathogen-free colonies of mice and guinea pigs.

As a result of participation in the workshop, a small-scale gnotobiotic program was initiated at the Fort Detrick Animal Farm. The program has three major objectives:

(a) To investigate the adaptability of existing gnotobiotic techniques to Animal Farm facilities,

(b) To improve the quality of the Animal Farm's specific pathogen free breeding colonies by providing a nucleus of gnotobiotic stock, and

(c) To provide a fund of experience in gnotobiotic technology for future use in these laboratories.

The basic gnotobiotic apparatus and methods initially employed were developed and reported by other investigators, notably R. Reyniers, Trexler and their associates at Lobund. Many of their apparatus and methods were adapted to our own facilities with only minor modifications.

However, effective sterilization of food proved a major problem. Contaminations occurred during our early attempts to establish and operate a gnotobiotic system. These cases of contamination were traced to inadequately sterilized food.

In this method originally employed, food in paper bags was placed in a metal can designed for use with germfree isolators. The cans and their contents then were steam sterilized in an autoclave, which had been evacuated to 28 inches of mercury pressure before steam was released into the chamber. Subsequent investigations showed that the length of time required to effect complete sterilization by this method would result in an unacceptable loss in the nutritive value of the food.

Appraisal of existing, alternative sterilization methods, such as irradiation or the processing and preparation of special foods, indicated that they were prohibitively expensive for the scope of our operations. Consequently, an investigation was undertaken to determine the possibility of evolving economical and effective apparatus and methods for the sterilization of food for gnotobiotic animals.

This paper is a preliminary report of the results of this investigation.

First, we will present a brief, general description of the basic apparatus and methods used in establishing our gnotobiotic system, including pertinent modifications made at Fort Detrick. Detailed descriptions of these basic apparatus and methods can be found in the original reports listed in the References.

Second, we will present a detailed description of the design, construction, and operation of an apparatus for the steam sterilization of food for gnotobiotic animals.

Finally, we will summarize our results to date and indicate the trends and objectives of future work.

II. GENERAL GNOTOBIOTIC APPARATUS AND METHODS

A. ISOLATORS

The animal isolators used in our investigations are modifications of those originally described by Trexler and Reynolds.[1]* Ours are fabricated locally from 20-mil polyvinyl chloride.** The basic unit measures 24 by 24 by 48 inches and is supported on a plywood frame. Accessories include standard one-piece neoprene gloves, an inlet air filter, a trap for outlet air, a pass-through lock, and a sterilization port. Front and rear views of the isolator are shown in Figures 1 and 2.

The isolator units are prepared for use in the following manner: First, the inlet of the air filter that attaches to the isolator is sealed off with a Mylar plastic film. Next, the filter is sterilized with dry heat at 150°C for two hours, then a polyvinyl chloride cover is placed on the filter.

The air filter and other accessories are attached to the basic unit. A spray gun is passed into the unit through the lock, an air pressure line is run into the unit via the sterilization port, and the entire interior of the unit is sprayed with a two per cent peracetic acid solution. The gun and air pressure line are removed from the unit, the pass-through lock and sterilization port are sprayed and sealed off, and the unit is allowed to stand overnight.

Next, an operator dons the gloves attached to the isolator and reaches across inside the unit and punctures the Mylar plastic film covering the inlet of the air filter. The air supply line is attached to the outside sleeve of the air filter, and the air is turned on. Positive air pressure is maintained within the unit throughout all subsequent operations. Following the 24 to 48 hours allowed for drying the interior of the unit, the isolator is ready for use.

B. AUTOCLAVE

The autoclave used in these studies consists of a conventional, 15-psig (22 psig maximum), jacketed, steel, pressure vessel with a door on one end. The autoclave has separate steam feed and condensate lines so that the jacket and chamber can be operated separately.

The autoclave has been modified by the attachment of a piston-type vacuum pump, which is used to reduce the chamber pressure to 28 inches of mercury. The purpose of the chamber vacuum is twofold: (a) to rapidly

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* See Literature Cited

** Cadillac Plastic and Chemical Company, Detroit, Michigan.

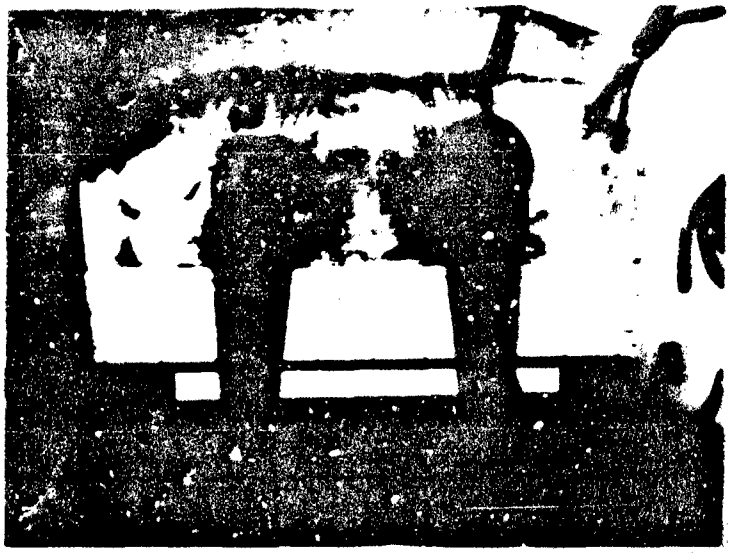

Figure 1. Front View of Plastic Isolator for Gnotobiotic Animals, Showing Attached Gloves. (FD Neg C-6154)

Figure 2. Rear View of Plastic Isolator for Gnotobiotic Animals Showing (left to right) Air Trap, Pass-Through Lock, Inlet-Air Filter with Air Supply Line Attached, and Sterilization Port on Right End. (FD Neg C-6151)

the chamber air so that the steam enters with a driving force that helps it to penetrate thoroughly the microorganically closed system being sterilized, and (b) to reduce the total time required for the chamber and items in it to reach sterilization temperature.

The general sterilization procedure is as follows: Fifteen minutes prior to a sterilization run, the jacket of the autoclave is preheated to reduce the chamber heat-up time. Then the items to be sterilized are placed in the chamber, the door is closed securely, the vacuum pump is started, and the chamber pressure is reduced to 28 inches of mercury. Once the proper pressure is reached, steam is released into the chamber, and the system is brought to 15 psig. The chamber condensate line is not opened until the chamber reaches atmospheric pressure. The sterilization time is measured from the time at which the chamber reaches 250°F.

When the sterilization cycle is complete, the chamber steam is shut off, and the contents are allowed to remain in the chamber for an additional 15 minutes to dry. A steam jet ejector on the autoclave partially evacuates the chamber and removes moisture during this part of the cycle.

C. STERILIZATION CANS

These cans, used for the sterilization of bedding, water, and food and their subsequent transfer to gnotobiotic isolators, have undergone considerable investigation during our studies. The can currently in use proved the best of three types investigated.

The same problem is basic to any can used for this purpose: When a can filled with bedding, water, or food is placed in the autoclave, it must be a microorganically closed unit. The can must have an effective microorganic barrier that not only will allow efficient sterilization of the can's interior and contents but also will keep the can closed to subsequent contamination following removal from the autoclave.

The basic can now being used is 24 inches long and has an inside diameter of 11½ inches, except at the open end, which flares in the last half inch to an outside diameter of 12 inches (Figure 3). The solid portions of the can are constructed of 18-gauge, Type 304 stainless steel. The central portion of the can is constructed of flattened diamond pattern, ½-inch by 18-gauge, stainless steel expanded metal, which provides an open surface area ranging between 65 and 70 per cent. The open, central portion of the can is 12 inches long.

A microorganic barrier is created over the central portion of the can with a blanket wrapping of four layers of Speed-Flo F-50 Fiberglas filter material.* This wrapping overlaps the solid portion of the can

* American Air Filter Company, Inc., Louisville, Kentucky.

Figure 3. Over-All View of Basic Sterilizer Can, Showing Expanded Metal Center Section. (FD Neg C-6310)

Figure 4. Sterilizer Can Ready for Sterilization, Showing Filter and Protective Covering Strapped in Place, Wire Mesh Metal Platform in Bottom of Can, and Mylar Plastic Covering Open End. (FD Neg C-6111)

by about two inches at each end. A protective layer of 14 by 18 mesh copper screening then is placed over the filter material and clamped securely in place with standard, ½-inch, flat steel strapping.

A platform, 5 inches wide by 23 inches long and constructed of the same material as the central portion of the can, is placed in the bottom of the can. This platform prevents condensed steam from saturating the contents of the can during sterilization. When a filled can is ready for sterilization, the open end is covered with Mylar plastic stretched over the flare and fastened with one-inch, transparent, pressure-sensitive plastic tape.* A can with the microorganic barrier, platform, and plastic covering all in place is shown in Figure 4.

D. GENERAL GNOTOBIOTIC REGIMEN

Isolator units were prepared to receive gnotobiotic stock in the manner previously described. Basic equipment to be used within the isolators, such as animal cages, were placed in the isolators and sterilized at the same time the isolators were sterilized.

Gnotobiotic mice were derived by Caesarean section, using the methods and techniques described by Reynie. s[2,3] and others. Methods and techniques used in the Caesarean derivation of gnotobiotic guinea pigs will be reported in another paper.

All Caesarean sections were performed in an isolator unit modified for the purpose. This isolator has a pair of glove ports on each side, and the pass-through lock is located at one end instead of at the rear. An access port and platform for holding pregnant females, similar to that described by Reyniers[2,3] and others, is located in the center of the base of the Caesarean isolator unit.

Caesarean-derived stock are transferred from the Caesarean isolator unit to a regular isolator in the following manner: The outside covers of the pass-through locks on both units are removed, and the locks are connected by a polyvinyl chloride sleeve held securely in place by pressure-sensitive tape. Rubber stoppers are removed from the two sterilization ports on the sleeve, and air in the sleeve is exhausted by collapsing the sleeve by hand. The sleeve then is reinflated with a mist of two per cent peracetic acid sprayed through the sterilization ports. The ports and stoppers are sprayed, the stoppers are replaced, and the units are allowed to stand for one-half hour, at which time the sleeve is considered to be sterile. The inside covers of the pass-through locks of both units then are removed, and the Caesarean-derived gnotobiotes are transferred to the regular isolators.

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* Scotch Brand No. 471, Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.

In practice, the two isolators are connected, and the sleeve is sterilized before the Caesarean is performed in order to keep the time between Caesarean derivation and transfer of the young to a minimum.

Originally, gnotobiotic mice provided by Lobund Institute were used as foster mothers for Caesarean-derived litters from our own breeding stock. Immediately following parturition by a Lobund female, Caesarean section was performed on a selected, parturient female from our own breeding stock. The litter in the isolator then was replaced by the litter derived by Caesarean section.

Food, bedding, and water used in our studies originally were sterilized in the autoclave and sterilizer cans previously described. However, investigations conducted following the contamination of some isolators showed that these apparatus and methods were unsatisfactory for food sterilization. The time periods required to sterilize the food effectively were in excess of the allowable limits for the heat-labile vitamin constituents of the diet being used. This problem led to the development of the food sterilization unit, which is described in Section III.

The autoclave and sterilizer cans still are used, however, for the routine sterilization of bedding, water, and miscellaneous equipment and instruments passed into our gnotobiotic system. All are sterilized at 250°F for 90 minutes, using the autoclave procedure previously described.

Following sterilization, the sterilizer can is connected to an isolator unit with a plastic sleeve identical to that described for the transfer of Caesarean-derived stock. Methods used for the sterilization of the connecting sleeve also are identical. When sterilization of the sleeve is complete, the operator dons the gloves on the isolator unit, removes the inside cover of the isolator pass-through lock, punctures the Mylar covering on the sterilizer can, and transfers the contents of the can to the interior of the isolator. The inside cover of the isolator pass-through lock is replaced, the connecting sleeve is detached, the outside cover of the pass-through lock is replaced, and the pass-through lock is sprayed, via its sterilization ports, with two per cent peracetic acid to insure sterility.

Figure 5 shows a sterilizer can connected to an isolator. Figure 6 shows a roll of the terry toweling used for guinea pig bedding being transferred to the interior of an isolator.

Our gnotobiotic studies now are about two and a half years old. The contaminations experienced early in the work apparently were caused by the inadequate sterilization of food. Since the completion of our detailed studies of sterilization techniques and the installation of the food sterilization unit described in the following section, no contaminations have been detected.

Figure 5. Sterilizer Can Connected to Plastic Isolator for Transfer of Sterilized Materials. (FD Neg C-6350)

Figure 6. Transfer of Sterile Bedding for Sterilizer Can into Plastic Isolator. (FD Neg C-6351)

III. DESIGN, CONSTRUCTION, AND OPERATION OF THE FOOD STERILIZATION UNIT

A. BACKGROUND

When it became apparent that the existing method of steam sterilizing food was inadequate, alternative sterilization methods were investigated.

Beta irradiation sterilization systems, such as those in use at the National Institutes of Health and elsewhere, were producing good results, but they proved too expensive for our purposes. However, one characteristic of such systems, the packaging of sterile food in sealed plastic bags, was adopted for use in the system we developed. Investigations of the processing and preparation of special diets within our gnotobiotic system also indicated that the costs would be prohibitive for the scope of our operations.

When the investigation of alternative methods indicated that further effort would be unprofitable, we decided to re-examine the steam sterilization method being used. Our purposes were threefold:

(a) To determine the reasons for the inadequacy of the existing method

(b) To compile data defining optimum sterilization conditions

(c) To use the data thus obtained to develop apparatus and methods for the economical and effective sterilization of food for use within our gnotobiotic system.

B. EVALUATION OF THE EXISTING METHOD

Although rather exhaustive tests were conducted during this evaluation, only the pertinent points are summarized here.

In order to measure the heat transfer characteristics of items processed in it, the autoclave previously described was modified so that four iron-constantan thermocouple leads could be inserted through the steam condensate line into the chamber. The leads were long enough to permit them to be inserted not only in material placed openly in the autoclave chamber but also in material contained in sterilizer cans placed in the chamber. A 12-point, continuous-recording, strip-chart recorder* was used as the indicating and recording system for the leads.

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* Model 153X60P12-X-111, 0-300°F, Minneapolis-Honeywell Regulator Company, Philadelphia, Pennsylvania.

The food* used in the tests was handled in two ways: (a) it was packed in Number 5 kraft bags, and (b) it was spread four inches deep in wire mesh baskets five inches wide by five inches deep by eight inches long. Food prepared in each manner was tested in the autoclave in each of the three types of sterilizer cans being investigated. In the tests with the sterilizer can previously described, autoclave tests were run both with and without vacuum. Tests also were conducted with food prepared in both ways and placed openly in the autoclave chamber; these tests also were run both with and without vacuum. In each test, the 12 points of the thermocouple system were distributed as follows: three each in the front, in the middle, and in the rear of the food load, and three in the autoclave chamber.

In all tests conducted under vacuum, the time required for the autoclave chamber temperature to reach 250°F ranged between three and five minutes. In the tests without vacuum, the time for chamber heat-up ranged between seven and nine minutes. Table I summarizes the findings with regard to the time required for the food load to reach sterilization temperature.

In brief, the tests showed that food, either in bags or baskets, could be sterilized in 35 to 45 minutes, using the Type 3 sterilizer can under vacuum in the autoclave. These time periods included an allowance of 20 minutes beyond the time that any given point in the food load reached sterilization temperature. Use of the Type 3 can without vacuum was rejected.

Although the Type 3 can seemed usable for food sterilization under these conditions, whether or not the food would have retained sufficient nutritive value remained questionable. The heat-labile vitamin constituents of the food undergo a marked reduction in strength when subjected to steam sterilization for more than 20 minutes, as shown by the manufacturer's analysis:

FOOD CONSTITUENT	PER CENT LOSS WHEN AUTOCLAVED FOR:[a] 20 Minutes	30 Minutes
Vitamin A	20	57
Thiamin	42	58
Riboflavin	0	0
Niacin	0	0
Pyridoxine	0	0
Pantothenic acid	15	34

a. Letter from Ralston Purina Company, September 18, 1961.

* Lab Chow Special Formula 2, Number 5010, Ralston Purina Company, St. Louis, Missouri.

TABLE I. MAXIMUM TIME FOR ANY GIVEN POINT IN THE FOOD LOAD TO REACH STERILIZATION TEMPERATURE (250°F)[a]

TEST CONDITIONS	TIME, Minutes	
	VACUUM	NO VACUUM
Type 1 Sterilizer Can		
Food in bags:	130.0[b]	----
Food in baskets:	82.5	----
Type 2 Sterilizer Can		
Food in bags:	34.7	----
Food in baskets:	25.6	----
Type 3 Sterilizer Can		
Food in bags:	19.8	31.0
Food in baskets:	17.6	21.1
Autoclave Chamber		
Food in Bags:	6.8	9.5
Food in baskets:	8.8	13.0

a. Where more than one run was completed, the figures represent the average for the particular thermocouple location.

b. Run was stopped at 130 minutes, at which time temperature had not reached 250°F at any point.

Consequently, even with the enriched-vitamin-content food being used to offset the effects of heat degradation, the sterilization times required in our tests with the Type 3 can seemed excessive. Without either a comprehensive chemical analysis of residual vitamin content as a function of sterilization time or a long-term trial in which the food was supplied to a control colony of germfree animals, firm conclusions regarding the nutritive value of the food were impossible.

However, the tests also showed that food, either in bags or baskets placed openly in the autoclave chamber, could be sterilized in a much shorter time with or without vacuum. Test results also indicated that the depth of the food had a definite effect on the time required for all of the food load to reach sterilization temperature. These two factors indicated two alternative methods for providing sterile food to our gnotobiotic stock:

(a) Sterilization of food either in bags or baskets, with or without vacuum, in autoclaves connected directly to each isolator, or

(b) Sterilization of food on passage into a sterile unit, where the food could be packaged in sterile containers, and then be removed from the unit aseptically for supply to any of the isolators.

From both a practical and an economical viewpoint, the second method was the method of choice, and the food sterilization unit now being used evolved from this concept.

C. CONSTRUCTION AND MAJOR COMPONENTS

In many respects, the food sterilization unit (Figure 7) resembles an isolator. The unit is a sealed, sterile enclosure with provisions for the pre-sterilization, entrance, processing, and aseptic removal of materials. Materials are sterilized in and introduced to the unit through an attached autoclave. Work is performed within the unit through standard neoprene gloves attached to the wall opposite the autoclave. Processed materials are removed through an aseptic dunk bath.

The basic chamber of the unit is constructed of extruded-sheet methyl methacrylate,* which is especially suitable for structures of this type. The plastic sheets were cut to size, and screw holes were drilled and countersunk. All joints were solvent-activated with chloroform for approximately 20 minutes, then pressed together and held firmly with the screws. Joints formed with this plastic in this manner are uniform, tough, transparent, leakproof, and structurally sound. If a joint is not transparent, it is a good indication that it is not structurally sound.

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* Plexiglas II, ½-inch, Rohm & Haas Company, Philadelphia, Pennsylvania.

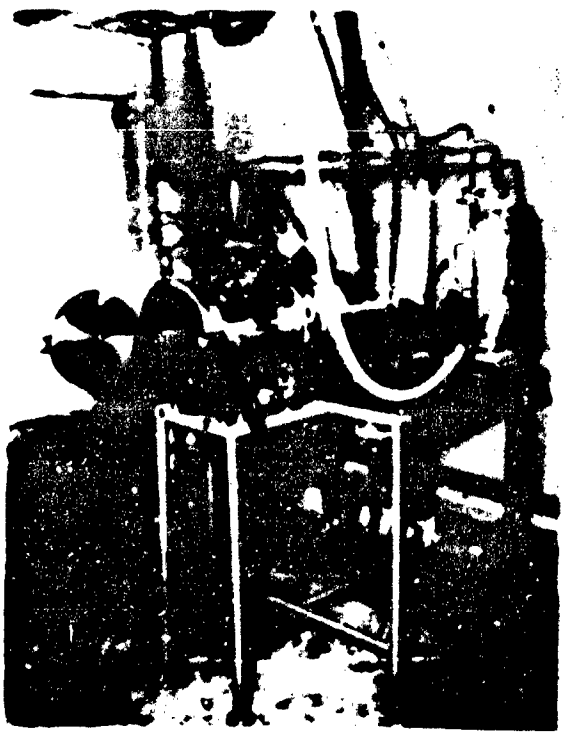

Figure 7. Over-All View of Food Sterilization Unit.
(FD Neg C-6296)

The dunk bath, drain trap, air filter and air trap supports, and all air pipes for the unit also were constructed of the same plastic and welded by the same techniques.

Where metal fittings were required in the walls of the basic unit, holes were bored and tapped for the proper thread size, and the fittings were sealed around the threads with two-part Epoxy cement.

Mineralite cable was used as the line carrier for the one electrical outlet required within the chamber. Mineralite cable is a copper-sheathed pipe containing electrical wires embedded in a hard-packed non-conductor, which prevents any microorganisms from traveling into the chamber through the pipe. Moisture-proof electrical receptacles were used both inside and outside the chamber.

The autoclave attached to the food sterilization unit is a double-doored, Keyniers-type autoclave with a chamber 16 inches long by 9 inches in diameter. The autoclave is attached to the plastic housing with a stainless steel connector plate, which has a water jacket around its outer front perimeter. Tap water circulating at a rate of 23 gallons per hour through the jacket maintains the adjoining plastic at 70° to 80°F when the autoclave is in operation. Because the melting point of the plastic is approximately 180°F, the water must be circulating at all times when the sterilizer is being used and for about two hours following shutdown. Two-part Epoxy cement was used as a sealer around the heads of the bolts connecting the autoclave to the connector plate. The same cement also was used as a sealer between the water jacket and the plastic housing.

As manufactured, no provision was made on the autoclave for operating the jacket steam irrespective of the chamber. Consequently, the autoclave had to be modified to provide separate steam condensate lines for the chamber and the jacket. It also was necessary to provide a steam bleeder line on the chamber steam-condensate line so that the chamber pressure could be bled to atmospheric with steam on the jacket. These modifications were necessary so that the food could be dried in the chamber following sterilization.

The inlet-air filter used on the unit is essentially a small version of the sterilizer can previously described. It consists of a stainless steel cylinder 18 inches long by 3 inches in diameter, with solid ends and a 13-inch central section made of the same stainless steel expanded metal used in the sterilizer can. The same filter material and protective copper mesh screen used on the sterilizer can also are used on the air filter. In addition, however, a polyvinyl chloride sleeve is placed over the protective screen before all are clamped firmly in place. Around the cylinder are adjustable lamps. The inlet-air filter is sterilized in the same manner previously described for the isolator inlet-air filters.

Air is fed into the filter through a tube in the polyvinyl sleeve. The air passes through the filter material into the cylinder and exits at the top of the cylinder through a one-inch stainless steel nipple. This nipple is connected to the air inlet of the food unit with a Tygon tube approximately three inches long and one inch in diameter. Air is provided to the unit at a flow rate of approximately four cubic feet per minute, which provides approximately one complete air change in the unit every three minutes. A positive air pressure equivalent to approximately 3/4 inch of water also is maintained in the unit at all times.

Originally, a glycerin air trap was used on the food unit air outlet. In such a trap, the outlet air pressure floats an inverted cup in a pool of glycerin as long as positive pressure exists. If the air pressure drops, the cup submerges and seals the exhaust, thus preventing a backflow of contaminated air into the unit. We found, however, that the hygroscopic nature of the glycerin caused the liquid level to rise in the trap. This build-up not only presents a contamination hazard, but also will result in blocking the trap if not attended regularly. Consequently, the glycerin was replaced with heavy mineral oil, which has given satisfactory results.

The dunk bath is located at the bottom of the food sterilization unit on the right side. The bath measures approximately 10 inches by 14 inches by 12 inches deep. A 7-inch extension of the side of the food chamber divides the open top of the bath so that half opens into the chamber and half opens to the outside of the unit. The bath is filled with two per cent Lysol solution. The bath allows sterile materials prepared in the unit to be passed to the outside without contaminating the unit. A lid is provided to cover the portion opening on the inside of the unit when the bath is not in use.

The last major basic component of the food sterilization unit is the drain trap. This consists of a square drain pit located to the left of center in the bottom of the unit. The drain pit empties into a U-tube trap, which is filled with Lysol solution. The drain trap makes it possible to remove spilled liquid wastes from the unit without dumping them into the dunk bath, which could spoil the dunk bath solution and necessitate a shut-down.

D. TESTING AND STERILIZATION

The unit was tested for leaks with a Freon Leak-Detection Apparatus. First, the chamber was evacuated to six inches of water vacuum. Then a container holding Freon under pressure was attached to the sterilization port, the vacuum on the chamber was released, the Freon refrigerant gas was fed to the chamber until the internal pressure reached six inches of water, and the chamber was sealed.

The leak detector, a hand-held, gun-type device, then was used to trace all perimeters and other possible leak points. Even trace quantities of leaking Freon will cause a deflection of the needle on the detector gauge and also cause the detector to emit an audible tone. One leak was found around the dunk bath in the first test. The leak was sealed with Epoxy cement, and the unit was retested and found to be free of leaks.

Once the food sterilization unit was proved leakproof, it was ready for sterilization and subsequent operation. The sterilization port consists of a double-valved, 3/8-inch, stainless steal pipe located in the right wall of the unit.

To sterilize the unit the outer door of the autoclave and all autoclave valves are closed securely. The inner door of the autoclave is opened, and the inner door ring gasket is removed. The chamber is evacuated to six inches of water vacuum. A container holding ethylene oxide gas under pressure is attached to the sterilization port. The vacuum on the chamber is released with the introduction of ethylene oxide, which is continued until the internal chamber pressure reaches six inches of water. The chamber then is sealed, and the unit is allowed to stand for 24 hours, after which all internal components are sterile.

As a check on sterility, a test strip containing spores of Bacillus subtilis var. niger was placed in a cotton-stoppered test tube in the chamber during sterilization. The strip was a part of the packaged standard sterility test material provided by an autoclave manufacturer.* The test strip proved negative, the control strip positive.

This sterilization procedure for the food unit also provides a secondary check of the tightness of the system. If any leaks are present, a drop in pressure will be noted at the end of the sterilization period, as indicated by a U-tube water manometer, which can be used to monitor pressure throughout the sterilization period.

E OPERATION

Once the unit has been sterilized, the basic methods of operation are relatively simple. Materials are sterilized in the autoclave, passed into the sterile chamber, sealed in sterile containers, and removed from the chamber via the dunk bath. Materials prepared in this manner then can be introduced into the isolators through the isolator pass-through locks, using the method of chemical sterilization previously described. Although the following description concentrates on the processing of food through the unit, preliminary tests have shown that bedding, water, and other materials to be used in the isolators can be processed through the sterilization unit in the same manner.

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* Sterilization efficiency test package, American Sterilizer Company, Erie, Pennsylvania

Food to be processed through the unit is placed in stainless steel wire mesh baskets 1½ inches deep by 5 inches wide by 11 inches long. The previous autoclave tests showed that this depth of food in wire baskets could be sterilized within the maximum allowable time limits. Two of these baskets full of food can be sterilized in the unit autoclave at one time.

Approximately 15 minutes before a sterilization run, the steam line to the autoclave jacket is opened to allow the jacket to preheat in order to reduce the chamber heat-up time. When the jacket temperature reaches 250°F, the two baskets of food are placed in the chamber, the door is closed securely, the chamber steam valve is opened, and the chamber is brought to 250°F (15 psig). This requires about five to seven minutes. When the chamber reaches sterilization temperature, it is maintained at sterilization temperature and pressure for 20 minutes. The chamber steam then is shut off, bled to atmospheric pressure, and the food is allowed to dry for 15 minutes. Following the drying period, the autoclave door to the interior of the unit is opened, and the sterilized food is removed from the autoclave into the unit (Figure 8).

The containers used for packaging the food are 8-inch by 12-inch, 20-mil, polyvinly chloride bags. These bags are pre-sealed on three sides and examined for flaws outside the unit. Paper towels are placed in and between the bags to keep them open and separated during sterilization. They then are sterilized in the autoclave for 20 minutes and dried before passage into the unit. The bags are allowed to cool in the unit before they are used because the autoclave heat makes them soft and pliable, and they tear easily in this state.

Figure 9 shows one of the bags being filled. Bags are filled with food to approximately 75 per cent of volume. The open end then is sealed with a heat sealer* contained in the unit. This sealer is preset to operate at 350°F and has a constant-temperature control to maintain the jaws at that temperature. The movable top jaw and the base of the sealer were covered with ½-inch blocks of Insurock** to prevent transfer of excessive heat to the chamber floor and the operator's work gloves.

Before the bags are sealed the end to be sealed is inspected visually to see that no food fragments are in a position to interfere with the seal. The open end then is placed between the jaws of the sealer.

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* Powerweld Crimpmaster, Model 251, 115-volt a.c., 500-watt, Cleveland Lathe & Machine Company, Cleveland, Ohio.

** The Richardson Company, Melrose Park, Illinois.

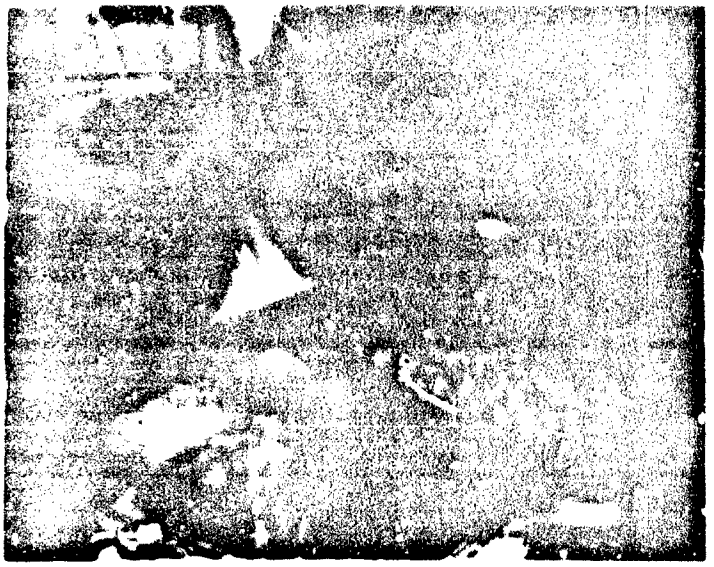

Figure 8. Sterilized Food Being Removed from Autoclave into Chamber. (FD Neg C-6293)

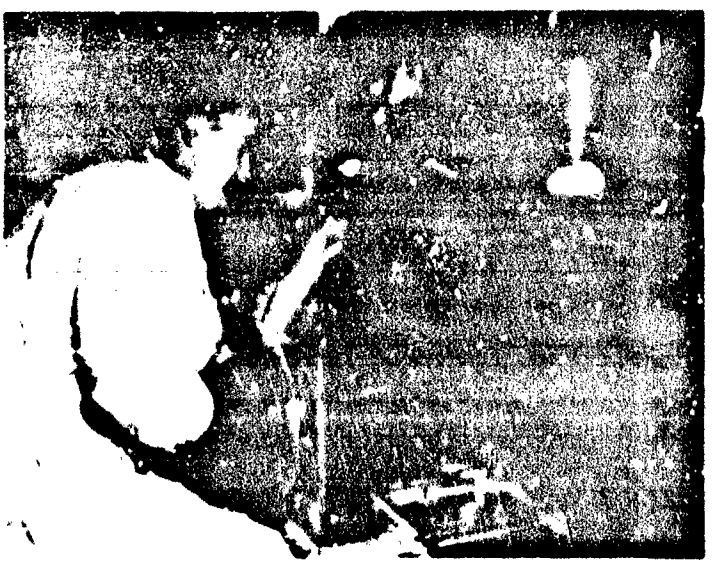

Figure 9. Operator Filling Polyvinyl Chloride Bag with Sterilized Food. (FD Neg C-6294)

The top, spring-loaded, movable jaw is pressed down by hand and held in position for five seconds. This sealing time is extremely critical because polyvinyl chloride film is not normally heat-sealed. Too much heat will melt the plastic, too little will result in an imperfect seal. Although the technique sounds difficult, it is actually quite simple once the timing is perfected. Figure 10 shows a bag being sealed.

After the bag is sealed, it is set aside to cool to room temperature. Then the bag may be removed from the unit via the dunk bath. The lid of the dunk bath is removed, the bag is pushed down under the dividing partition of the bath, and the buoyancy of the air sealed in the bag causes it to float to the surface on the outside. The bag can be submerged in the dunk bath solution and squeezed to check for leaks. After this, the bag can be stored for future use or transferred immediately to an animal isolator. Figure 11 shows a sealed bag being placed in the dunk bath

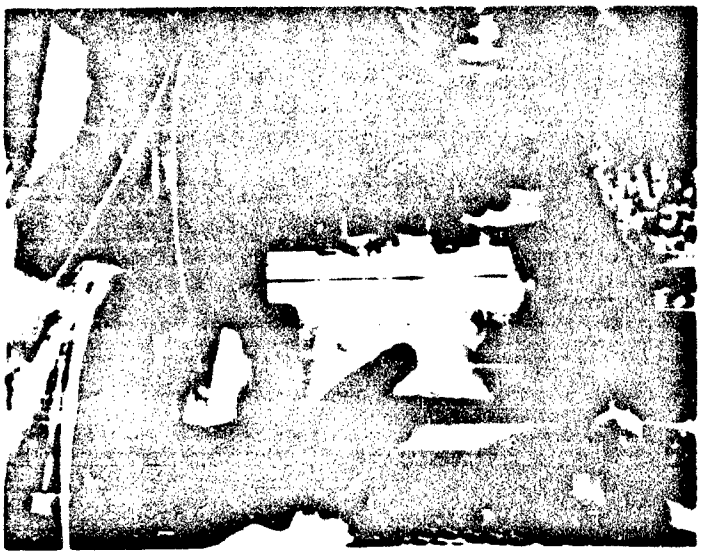

Figure 10. Bag Filled with Sterile Feed Being Heat-Sealed. (FD Neg C-6295)

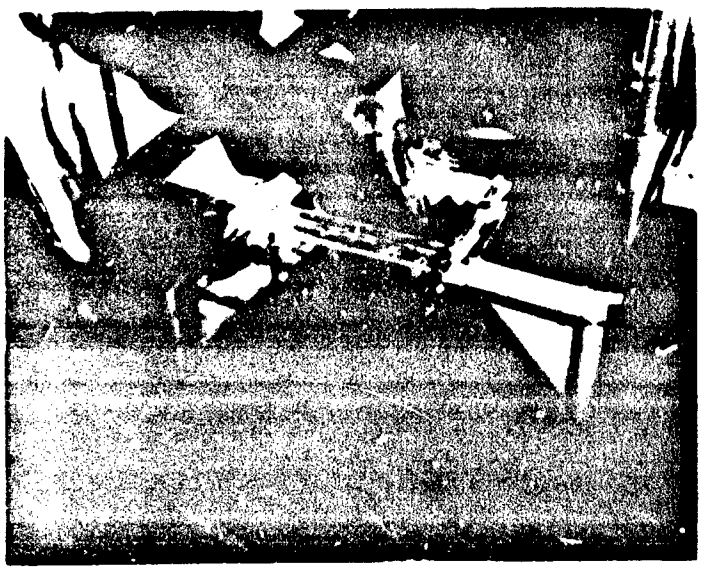

Figure 11. Sealed Bag of Sterile Feed Being Placed in Dunk Bath for Transfer to Outside of Unit. (FD Neg C-6294)

IV. DISCUSSION

The sterilization of food for germfree animals presents a serious problem to the investigator who cannot afford to make a large investment in equipment. The only method previously available at nominal cost was the one recommended at the 1961 Lobund Gnotobiotic Workshop, which consisted of the basic regimen with the vacuum autoclave and sterilization cans previously described.

Extensive tests of this method in our facilities showed that steam sterilization for 45 minutes at 250°F was required to insure sterility of food in the apparatus used. Such a sterilization period is detrimental even to enriched feed because the heat-labile vitamin contents degrade rapidly when exposed to 250°F for more than 30 minutes. A survey of other alternative methods indicated that they were too expensive for our purposes. Consequently, we developed the food sterilization unit and methods described in the preceding section.

Since August 8, 1961, no accidental contaminations have occurred in our isolators. The food sterilization unit has been in operation for the past five months and has been used regularly to prepare food for the isolators throughout that time. Thirty-three entries have been made to the isolators in supplying food prepared in the unit.

The isolators have been checked periodically for contamination. Swabs of fecal material removed from the isolators are streaked on blood agar and inoculated in thioglycolate. These media are incubated at 37°C for one week. To date, no growth has been observed.

These negative cultures do not entirely rule out the possibility of contamination. However, they are a good indication of the effectiveness of the system because a major deficiency would have introduced organisms capable of growing on these media. Unfortunately, time has not yet permitted extensive bacteriological screening of the mice to determine more accurately their gnotobiotic status.

The sterilization time of 20 minutes used with our system is considerably shorter than those used in sterilizer can systems for the steam sterilization of food for gnotobiotes. This reduction in sterilization time not only increased the efficiency of the operation but also resulted in a higher quality, more palatable food. The charred appearance characteristic of food autoclaved for longer periods is not nearly as pronounced in our food. Furthermore, mice raised on food prepared in the unit have shown a significantly higher rate of reproduction than those raised on food processed in the sterilizer cans.

Actually, the question of the degradation of the nutritive value of food sterilized for supply to gnotobiotic animals remains a matter worthy of definitive investigation. To our knowledge, no detailed work has been

done on a comparison of the residual nutritive value of food sterilized by heat and other methods, such as irradiation. Long-term trials with gnotobiotic colonies raised on food sterilized by various existing methods could provide invaluable basic reference data on diets for use in future gnotobiotic investigations.

The total cost of the food sterilization unit described here was approximately $2500. However, this figure includes the cost of the initial engineering design work, including the modifications required in the autoclave. The cost to reproduce the food sterilization unit, including materials, construction, installation, autoclave, and all components, should not exceed $2000. This is relatively inexpensive when compared with other equipment capable of supplying the amount and quality of diet that can be produced using this system. The isolators we are using, including the basic plastic envelope, all attachments, and the base, cost approximately $200 each.

The fact that the single food unit can be used to prepare food for supply to a number of isolators by the described methods is one of the major factors in its economic feasibility. However, the single food preparation unit concept does present the possibility that an undetected contamination in the unit could affect many isolators. To date, we have encountered no difficulties, but frequent sterility checks are run on food prepared in the unit and extreme care is taken to prevent accidental contamination of the chamber while the unit is in operation.

Currently, we are storing food prepared in the unit within the unit chamber until it is needed for transfer to the isolators. When time studies of food prepared in the unit and then stored outside prove the invulnerability of the food package, food can be prepared in large batches and stored outside the unit until needed. Such outside storage will increase both the efficiency and economy of the system.

The ultimate solution to the problem of providing sterile food for gnotobiotic studies lies with the commercial feed manufacturers. When one of these produces a uniform, high-quality, sterile feed, the cost of laboratory studies with gnotobiotes will be reduced substantially.

V. RESULTS

Although our work with gnotobiotic animals has covered only a relatively short period, the results achieved to date have been very encouraging. One of the purposes in undertaking these studies was to improve the breeding stock at the Fort Detrick Animal Farm. Results to date indicate that worthwhile improvements are possible.

As noted previously, germfree mice supplied by Lobund Institute originally were used as foster mothers for Caesarean-derived mice from our own stock. Mice so raised in our gnotobiotic isolators were allowed to breed and reproduce, and, at three weeks, the weanling mice were transferred to a conventional clean breeding room set aside for these studies. The construction, use, and regimen enforced in such rooms at the Fort Detrick Animal Farm have been described in detail by Rabstein.[4]

Unquestionably, the germfree mice transferred to the conventional breeding room have since been contaminated with microorganisms. A systematic examination of these mice for known mouse pathogens is under way. Thus far, laboratory tests have shown that these mice are free of Salmonella, lymphocytic choriomeningitis, and Tyler's virus, which were eliminated from our conventional colonies in earlier work with Caesarean-derivation of breeding stock. Moreover, to date these mice have remained free of any clinical evidence of infantile diarrhea, which has caused occasional problems in our conventional colonies. Finally, these gnotobiotic mice also have remained free of ecto- and endoparasites. If this record can be maintained as this nucleus of gnotobiotic stock enlarges, it will effect a marked improvement in the quality of our mouse colony.

In addition to our gnotobiotic studies with mice, much effort has been concentrated on the establishment of a nucleus gnotobiotic stock of guinea pigs. A detailed report of this work will appear later. It may be of interest to note, however, that the problems involved in transition of germfree guinea pigs to the conventional state have proved much more complex than those in mice.

VI. CONCLUSION

The three goals established when we began our gnotobiotic studies show promise of achievement.

First, we have successfully adapted existing gnotobiotic apparatus and methods to our own facilities. The development of the food sterilization unit and attendant methods for its use, together with the modifications of other existing gnotobiotic apparatus and methods, have resulted in an effective gnotobiotic system that is economically feasible for the scope of our operations.

Second, the nucleus of gnotobiotic mice and guinea pigs successfully produced thus far will provide a means for improving the quality of our large-scale breeding colonies. As more experience in gnotobiotic apparatus and methods is gained, these techniques can be applied to the improvement of other species of laboratory animals.

Third, the experience we have gained in our studies will provide a fund of basic gnotobiotic information for other investigators in these laboratories.

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